Manuscript submitted to
Aims Environmental Science

Volume 1, 2013, 1-11.
DOI: 10.3934/environsci.2013.1.1

Received date 29 Jun 2013, Accepted date 23 Aug 2013, Published date 27 Sep 2013

Research Article

The effect of light and nitrogen availability on the caffeine, theophylline and allantoin contents in the leaves of *Coffea arabica* L.

Marcelo F. Pompelli [1,*], Gilmara M. Pompelli [1], Antônio F. M. de Oliveira [2] and Werner C. Antunes [3]

[1] Plant Physiology Lab, Department of Botany, Federal University of Pernambuco, CCB, Recife, Pernambuco, 50670901, Brazil.
[2] Applied Ecology and Phytochemistry Lab, Federal University of Pernambuco, Recife, Pernambuco, Brazil.
[3] Plant Physiology Lab, Department of Biology, Maringá State University, Maringá, Paraná, Brazil.

* **Correspondence:** E-mail: mfpompelli@gmail.com; Tel: +55-81-2126-8844; fax +55-81-2126-7803.

Abstract: Caffeine is the most abundant and important purine alkaloid derived from several important crop, such as coffee, tea, cocoa, guarana, and other plants. In tea and coffee plants, caffeine is predominantly produced in the young buds of leaves and in immature fruits. The effect of light-stimulating caffeine biosynthesis is uncertain, but our results clearly show that light, independent of N-availability, increases caffeine (26%), allantoin (47%), and theophylline (8%) content in plants compared with those grown in a shaded (50% solar irradiation) environment. Caffeine is the major low-molecular-weight nitrogenous compound in coffee plants, and at times, it functions as a chemical defense for new bud leaves. Therefore, the primary question that remains is whether caffeine can serve as a nitrogen source for other metabolic pathways. If so, plants grown under a low nitrogen concentration should promote caffeine degradation, with the consequent use of nitrogen atoms (e.g., in NH_3) for the construction of other nitrogen compounds that are used for the plant's metabolism. Our results provide strong evidence that caffeine is degraded into allantoin at low rates in N-deficient plants but not in N-enriched ones. By contrast, this degradation may represent a significant N-source in N-deficient plants.

Keywords: *Coffea arabica* L.; nitrogen compounds; nitrogen starvation; sun and shade plants

Abbreviations list

CNB = carbon/nutrient balance; N = nitrogen; HL = high light, full sunlight; CS = caffeine synthase; LN = low nitrogen, 0 mM; HN = high nitrogen, 23 mM; IN = intermediary nitrogen, 16 mM; LL = low light, shade; GS = glutamine synthetase, EC 6.3.1.2; ALN = Allantoin; N-ALN = nitrogenous allocated in the allantoin molecules

1. Introduction

Coffee is a major agricultural commodity in the world, and its production is economically important to several tropical countries, including Brazil, Vietnam, Colombia, Indonesia, Mexico, Ethiopia and Kenya, among others [1]. *Coffea arabica* L. (Rubiaceae) is the most cultivated and consumed coffee species in the world, yielding approximately 70 billion dollars per annum [2].

Caffeine is the most abundant and important alkaloid in coffee plants [3]. Although caffeine was first isolated from tea and coffee in the early 1820s, the biosynthetic and catabolic pathways of caffeine were not fully established until 2000 [4]. In tea and coffee plants, caffeine is predominantly produced in young buds, leaves [3,5,6] and immature fruits [7], and it continues to gradually accumulate during the maturation of these organs. Caffeine, as a product of secondary metabolism, is derived from purine nucleotides [8], which have been detected in nearly 100 species [9]. Strong evidence shows that caffeine is slowly catabolized by the removal of its three methyl groups, resulting in the formation of xanthine, which, in turn, results in considerable caffeine content in older and senescent leaves [10]. When catabolized, xanthine is further converted into theophylline, followed by the formation of 3-methylxanthine, which is catalyzed by N7-demethylase and N1-demethylase, respectively. This process is followed by the formation of uric acid, allantoin and allantoate, which are ultimately degraded into CO_2 and NH_3 [3,10,11].

Caffeine concentrations vary in plant tissues and throughout the plant's organs. The caffeine content of seeds derived from different *Coffea* species varies within the range of 0.4% to 2.4% [9]. In *C. arabica* seedlings, caffeine resides predominantly in the leaves and cotyledons at concentrations varying from 0.8% to 1.9% dry weight [6]. Currently, few environmental/physiological features have been shown to affect caffeine biosynthesis in the leaves or coffee seeds. Particularly, nitrogen (N) availability [12,13] and tissue age ([9] and references therein) have little impact on caffeine production. Nevertheless, little is known about the influence of abiotic factors on the biosynthesis of caffeine and its degradation in leaves. These studies suggests that, in coffee, caffeine is strongly regulated by genetic factors, and the environment is likely less important to the caffeine phenotype [13].

The coffee plant is adapted to the understory of tropical rainforests in its native habitat. Hence, the effect of light on caffeine biosynthesis is uncertain. Recent studies have shown that shaded or dark growth conditions generally increase the caffeine levels in tea leaves ([9] and references therein) or coffee cell cultures [14]. In contrast, light strongly stimulates the methylation steps of caffeine biosynthesis in the pericarp of coffee *(Coffea arabica)* [5,15]. Independent of this effect, light provides the energy to maintain elevated growth rates through photosynthesis and appears to provide a fixed carbon source for the increased production of primary metabolites, such as D-ribose 5-phosphate, which serve as substrates for caffeine biosynthesis via their conversion to purine nucleotides. Coffee plants growing in full sunlight exhibit an increase in their absolute and relative growth rates and process a higher leaf fraction [16]; i.e., a high carbon and nitrogen investment to build those leaves. On this favorable condition, high caffeine content helps protect the plant against herbivores [17]. According to the carbon/nutrient balance (CNB) hypothesis [18], the excess carbohydrates, especially starch, that accumulate in nutrient-rich plants [19] supply the energy and carbon skeletons necessary for secondary metabolism. Because alkaloids are nitrogen-containing compounds, lower caffeine content might be expected in nutrient-deficient plants, even for those with high carbon availability. Therefore, the production of nitrogen-based secondary metabolites (e.g., alkaloids) should increase as nitrogen (N) is acquired to supply primary metabolism and growth requirements. Additional N should then stimulate downstream metabolite biosynthesis [20].

Although there are a small number of exceptions, several reports have shown an increase in alkaloid content due to N fertilization [12,13]. In contrast, this response may vary depending on the nutrient source (nitrate, ammonium or urea) and based on the alkaloid type [21].

Caffeine may be degraded via the release of all four N atoms as ammonium [7]. Mazzafera and Gonçalves [22] detected caffeine and ureides in the xylem sap of *C. arabica* and suggested that the ureides may play a certain role in the N transport (and source) of this species; however, it appears that this usage is limited to certain tissues and to specific developmental stages. Because the coffee plant accumulates caffeine as a N sink and theophylline and allantoin as products of caffeine degradation, we investigated the role of light and nitrogen availability in regulating the caffeine, theophylline and allantoin content of coffee leaves.

2. Materials and Method

2.1. Plant materials and experimental design

The experiment was conducted in a greenhouse located at the Federal University of Viçosa (20º45'S, 42º54'W; 650 m a.s.l.) in southeastern Brazil. Uniform seedlings of coffee (*Coffea arabica* L. cv 'Red Catuaí IAC 44') obtained from the seeds [23], were transplanted into 12-L containers containing a mixture of soil and sand (2:1, v/v). After transplantation, the seedlings were randomly submitted to two light treatments, i.e., plants received either 100% or 50% solar irradiation, hereafter referred to as high light (HL) or low light (LL), respectively. During the experiment period, the maximum solar radiation reached more than 2000 μmol photons m^{-2} s^{-1} of photosynthetic active radiation (PAR). After 50 days in the different light environments, the plants were fertilized once per week with 250 mL of Hoagland's solution [24] containing 0, 16 or 23 mM nitrogen (hereafter referred to as LN, IN and HN plants, respectively), in the form of NH_4^+ or NO_3^-. The nutrient solutions were supplied until the LN plants, particularly the older leaves, exhibited visual symptoms of N deficiency [25]. The youngest, fully expanded leaves, corresponding to the third or fourth pair from the apex of plagiotropic branches, were then sampled and measurements were performed. The experiment consisted of a completely randomized design with 6 treatment combinations forming a 2 × 3 factorial (2 light and 3 N levels), with 7 plants in individual pots per treatment combination as replications. The experimental plot shows one plant per container. The experiments were repeated, and they produced similar results.

2.2. Caffeine content

The caffeine content was measured as described in [6], with some modifications. The coffee leaves were dried in a hot air oven at 70 ºC at constant weight and then ground in 80% methanol (v/v) using a mortar and pestle. After incubation at 60 ºC for 30 min, the homogenates were centrifuged at 12,000 × *g* for 10 min (25 ºC), and the supernatant was collected. After the complete evaporation of the methanol in a speed-vac, the methanol-soluble extracts were dissolved in distilled water. The samples were filtered (0.45 μm filters) using a disposable syringe filter unit (DISMIC-3CP, Advantec, Tokyo, Japan) and then injected into an HPLC (Shimadzu LC 10A HPLC system; Shimadzu, Kyoto, Japan). The mobile phase was MeOH:H_2O (4:1 v/v) at a flow rate of 1 mL min^{-1}. The separation was achieved using a C-18 column (4.6 × 250 mm) with 5-μm packing (Zorbax, model Eclipse XDB-C18). The effluent was monitored using a variable wavelength UV detector at 273 nm. Identification and quantification were performed at room temperature (10 μL of sample) using a reference standard (Sigma-Aldrich Chemical Co.).

2.3. Theophylline and allantoin content

To measure the theophylline and allantoin content, the *C. arabica* leaves were ground, and a methanol extract was obtained as described above. To quantify the xanthines, the method described by Filippi [26] was utilized.

2.4. Other biochemical measurements

Free amino acids were assayed using the ninhydrin method [27]. Soluble protein concentrations were determined using Bradford's method [28] with BSA as a standard. The total N was estimated as the sum of the ammonium-N and nitrate-N pools [23]. Glutamine synthetase (GS; EC 6.3.1.2) activity was measured by tracking the production of γ-glutamylhydroxamate at 530 nm [29]. One unit (U) of GS was defined as the amount of enzyme required to produce 1 mmol of γ-glutamylhydroxamate per hour.

2.5. Statistical analysis

The data were statistically examined using a fixed-model ANOVA that followed a completely randomized design, and significant differences between treatments were analyzed using the Newman-Keuls test at $p \leq 0.05$. Mean comparisons were performed using Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA).

3. Results and Discussion

The effect of light and N availability on caffeine, theophylline and allantoin are shown in Table 1. On average, exposing the plants to high light (HL) conditions significantly ($p \leq 0.05$) increased the caffeine (26%), allantoin (47%), and theophylline (8%) content. The caffeine content of the HL plants was higher compared to the values previously reported [22]. However, these values are in agreement with previously reported concentrations for coffee leaves [6,9]. Light is an important environmental parameter that drives photosynthesis and regulates plant growth and development. It has been demonstrated that the caffeine content can increase [5,30], decrease [31,32] or remain unchanged [33] when plants are cultivated in HL environments. The mechanism by which light regulates caffeine biosynthesis has been demonstrated to an extent in some model plants. Light does not directly influence the rate of caffeine biosynthesis or directly affect the level of caffeine synthase (CS) in tea leaves [33]. However, CS, which catalyzes the final two steps of caffeine biosynthesis, is located in the chloroplasts [34] and has an optimal alkaline pH (8.5) that is similar to the pH of most light-activated photosynthetic enzymes [35]. Thus, light raises the stromal pH [36], which could indirectly stimulate CS activity.

Several studies have reported that theophylline could be involved in caffeine breakdown [3,4,7,9]. Significant catabolism of purine alkaloids was observed in both developing, intermediate-sized and fully developed leaves, in which [8^{14}C] theobromine was degraded to CO_2 via 3-methylxanthine, xanthine and allantoic acid [4,11].

Regardless of the light conditions, the leaf N concentration was, on average, 58% higher in the HN plants (≈30 g kg^{-1} DW) compared to the LN plants (≈20 g kg^{-1} DW; Table 1). The increase in total N was accompanied by an increase in the concentrations of N-NH_4^+ and N-NO_3^- (Table 1). Because N concentrations below 23 g kg^{-1} have been shown to induce visual symptoms of N deficiency in coffee [25], the LN plants were presumably N deficient. Other N deficiency symptoms, such as a decreased total leaf area or chlorophyll breakdown, were also observed in the LN plants

(data not shown). Therefore, the effect of N availability on the caffeine content was more pronounced in the IN plants than in the LN plants (≈117%) and consequently more pronounced in the HN than in the IN plants (≈11%); a sharp increase in the caffeine levels from the N-deficient to N-sufficient plants was observed, but not for the N-sufficient to N- enriched plants.

Table 1. Nitrogen allocation traits [$N\text{-}NH_4^+$, $N\text{-}NO_3^-$, total nitrogen, amino acids, glutamine synthetase (GS) activity, and caffeine, theophylline and allantoin content], in the leaves of coffee plants (*Coffea arabica* L.) under high light or low light and submitted to LN (0 mM), IN (16 mM) or HN (23 mM) levels of nitrogen. Different upper case letters represent the statistical significance between the means for each nitrogen level and different lower case letters represent the statistical significance among the means for each light condition ($p \leq 0.05$, Newman–Keuls' test). $n = 7$.

Parameters	High light			Low light		
	LN	IN	HN	LN	IN	HN
Caffeine (mmol kg^{-1} DW)	33.8 ± 1.2 Ca	70.4 ± 2.3 Ba	83.9 ± 3.6 Aa	26.7 ± 1.1 Bb	60.7 ± 2.2 Ab	61.8 ± 3.3 Ab
Theophylline (mmol kg^{-1} DW)	1.8 ± 0.2 Aa	1.4 ± 0.1 Ba	0.6 ± 0.1 Ca	1.3 ± 0.1 Ab	1.5 ± 0.1 Aa	0.7 ± 0.1 Ba
Allantoin (mmol kg^{-1} DW)	4.5 ± 0.3 Aa	3.18 ± 0.1 Ba	0.8 ± 0.1 Ca	3.7 ± 0.1 Ab	1.6 ± 0.1 Bb	0.4 ± 0.1 Ca
$N\text{-}NH_4^+$ (g kg^{-1} DW)	18.8 ± 0.9 Ca	25.7 ± 1.0 Ba	31.5 ± 1,0 Aa	20.6 ± 0.7 Ba	19.2 ± 1.5 Bb	26.4 ± 1.1 Ab
$N\text{-}NO_3^-$ (g kg^{-1} DW)	1.8 ± 0.1 Aa	1.9 ± 0.1 Aa	2.1 ± 0.1 Ab	1.6 ± 0.1 Ca	2.1 ± 0.3 Ba	2.7 ± 0.2 Aa
Total N (g kg^{-1} DW)	20.6 ± 0.9 Ca	27.6 ± 1.0 Ba	33.6 ± 1.1 Aa	20.0 ± 0.8 Ba	21.3 ± 1.6 Bb	30.4 ± 1.2 Ab
Amino acids (mmol kg^{-1} DW)	41.8 ± 4.9 Aa	42.0 ± 3.8 Aa	60.9 ± 4.6 Aa	47.8 ± 7.8 Aa	47.7 ± 7.8 Aa	55.7 ± 7.3 Aa
Total protein (g kg^{-1} DW)	116.7 ± 6.1 Ca	202.8 ± 3.4 Ba	283.0 ± 8.1 Aa	87.0 ± 4.6 Ca	160.7 ± 8.4 Bb	215.9 ± 8.9 Aa
GS (U kg^{-1} DW)	20.7 ± 1.4 Ba	42.6 ± 3.4 Aa	44.7 ± 3.1 Aa	15.9 ± 1.1 Ba	29.5 ± 1.0 Ab	34.6 ± 2.6 Ab

With respect to the nutrient-deficient plants, their low caffeine content may be a consequence of a lower N availability. Nevertheless, we suggest that caffeine is degraded into theophylline and other N-products [3] to supply N in N-deficient plants. Our results are consistent with Gonthier et al. [13], who described the effect of N fertilization on caffeine production in coffee plants and demonstrated an increase in the caffeine concentration in the phloem exudates as N fertilization increased. However, Gonthier et al. [13] described only an increase in caffeine levels as a consequence of the increase in nitrogen nutrition without any inference from the remobilization or degradation of caffeine in N-deficient plants, as shown in this study (Table 1; Fig. 1). As the available N increased in the HN plants, the theophylline and allantoin concentration decreased linearly (Fig. 1), and the LN plants displayed three and seven times higher theophylline and allantoin concentrations than the HN plants, respectively (Table 1). Another explanation for the reduction in the caffeine levels is that a possible reduction in the rate of caffeine biosynthesis occurs in nutrient-deficient plants, i.e., the reduction could be preceded by a decrease in the concentration of a caffeine precursor compound that broadly affects CS activity in vivo. The biosynthesis of N-containing secondary metabolites (e.g., caffeine) is very sensitive to the N levels of leaves [12]. In fact, the carbon/energy availabilities and the N content in the leaves alone may be insufficient for predicting resource allocation to particular secondary metabolites because their production is influenced by nitrate-induced signals and not merely by the mass flow of carbon and N through various metabolic pathways [37]. The high allocation of carbon, energy and N to caffeine during high N fertilization suggests that caffeine also serves as a storage metabolite for the N that is accumulated in excess of the plant's immediate need [38].

Significant catabolism of purine alkaloids was found in fully developed *Cocoa* sp. leaves at the same time that no biosynthesis was observed in older ones [11]. Thus, we propose that caffeine biosynthesis may be reduced in nutrient-deficient plants, with the caffeine produced in young leaves predominantly degraded in mature plants and the N recycled through different metabolic pathways, whereas the caffeine levels in N-deficient leaves were lower than those in the N-supplied leaves, and the caffeine catabolites were higher than the same counterparts (Table 1). Indeed, the extent to which caffeine accumulation is processed in leaves is still somewhat unclear. Any metabolite pool depends on its rate of biosynthesis and degradation or use. A reduction of caffeine biosynthesis in N-deficient plants seems doubtful given that caffeine biosynthesis occurs in higher rates in new, expanding leaves ([9] and references therein) and N-deficient plants always remobilize their N-pool from older leaves to new expanding ones. In other words, new leaves often are highly N-supplied [39], even in N-deficient plants. Thus, we expect that caffeine biosynthesis is less affected in the new leaves of LN plants but more affected in mature and older leaves and lower amount of caffeine content could be the result of its catabolism. The accumulation of caffeine degradation products (Fig. 1) strongly suggests that caffeine can be degraded and N recycled.

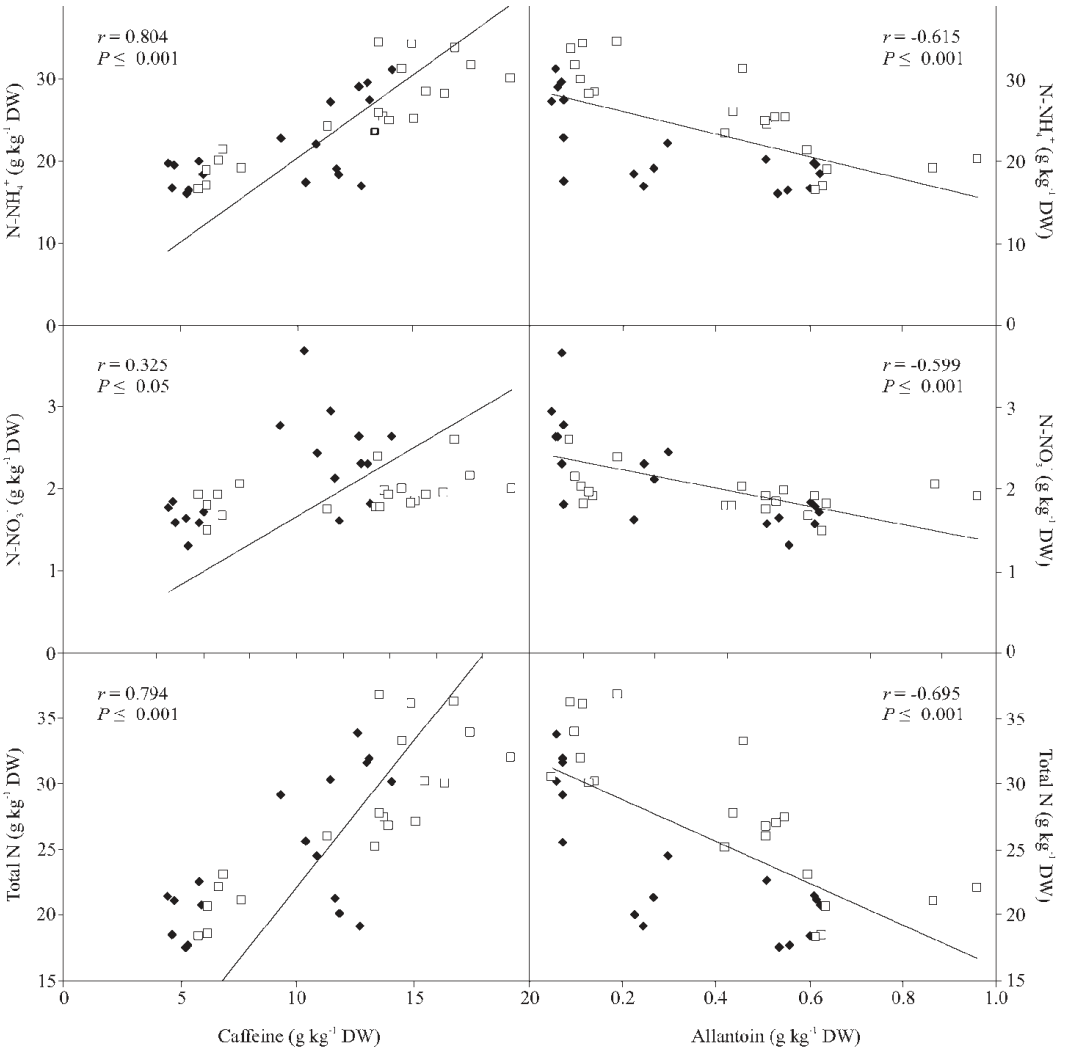

Figure 1. The relationship between caffeine, allantoin content and N-NH_4^+, N-NO_3^- and total N in the leaves of coffee plants (*Coffea arabica* L.) under high light (open symbols) or low light (dark symbols). *n* = 7.

Light had little effect on the free amino acids, glutamine synthetase activity, and total protein. However, the total protein and GS activity increased approximately 145% and 117%, respectively, in the HN plants compared with those in the LN plants. In addition, the free amino acid content increased ≈30% in the HN leaves compared to their LN counterparts. Nitrate reductase, an enzyme that catalyzes the reduction of nitrate to nitrite, is a very important part of amino acids synthesis, and it is mainly regulated by nitrate availability and light, which can vary in response to the light conditions and shows circadian or ultracircadian fluctuations [40]. In this respect, the light induced an increased in the leaf nitrate reductase activity in young coffee leaves [41], reaching maximum values between two and six hours after the beginning of the illumination period and decreasing thereafter until the end of the dark period. Moreover, Carelli et al. [42] showed that plants grown under full sunlight presented lower nitrate reductase activity. However, the inherent ability of coffee plants to acclimate to different irradiance regimes [2] might be the cause, at least in part, for the conflicting data in the literature concerning the influence of light on carbon assimilation. Conversely, it was discovered that the activity of GS, an enzyme that is thought to catalyze the rate-limiting step of photorespiration, was strongly increased after light treatment [23,43].

Caffeine contains four N-atoms, representing approximately 29% of its molecular mass. In young tissues, caffeine can accumulate at levels corresponding to 4% of the dry weight of the plant, indicating that approximately 1.15% of the DW contains N allocated only caffeine. Thus, approximately 15% of the respired carbon must be devoted to caffeine synthesis [5]. Previous studies have reported that N-containing compounds, such as alkaloids, might play a role as N reserve molecules [13]. However, considerable levels of caffeine remain in the leaves after abscission. Thus, caffeine does not appear to act as a nitrogen reserve [9]. Moreover, in *C. arabica* and *C. dewevrei* species, aged leaves and mature fruits displayed a lower capacity to degrade caffeine [10]. In N starvation experiments, Waller et al. [44] described how the caffeine content was preserved, indicating that the alkaloid is not easily utilized either for energy or as a nitrogen source. In contrast, Palumbo and co-workers [12] determined that the caffeine concentrations of *Ilex vomitoria* in the leaf tissues increased with N fertilization, suggesting that caffeine allocation is influenced by environmental factors in certain plant species. Whether caffeine can serve as an effective carbon and N source for coffee plants is especially unclear for N-supplied plants. However, caffeine might be used as a carbon source by some microorganisms [45,46]. Moreover, some alkaloids (i.e., theophylline, 3-methylxanthine and xanthine) may be utilized for the *de novo* synthesis of caffeine in young leaves [9].

Although caffeine has been detected in the xylem sap of coffee plants [22], its contribution to N transport when compared to nitrate and amino acids is negligible. Allantoin (ALN) is formed during caffeine degradation [3,4,10], but the major pathway for allantoin formation is the conventional catabolic pathway for purine nucleotides, which is also present in coffee plants [47]. However, experiments using radiolabeled tracers have unequivocally demonstrated that ALN is actively degraded during caffeine catabolism in coffee [10]. Filippi et al. [26] were not able to detect allantoinase activity in coffee plants, and they showed that when ALN was the exclusive source of N, the fresh mass of coffee cells comprised only a third of the amount found in the coffee plants subjected to ALN-free medium; this suggests that ALN might have been toxic to the cells. Unlike Filippi et al. [26], our study showed that the ALN level increased as the plant experienced N starvation. However, one would expect that the nutrient-deficient plants' demand for primary metabolism is high. The same pattern has been described by other authors [10,26]. Together with the protein and amino acid contents, our results indicate that despite ALN being taken up by the cells, the N-ALN was apparently not incorporated into amino-N compounds, as previously reported for

coffee cells *in vitro* (for more details on the biosynthesis and degradation of caffeine, see [3]). Altogether, these results provide strong evidence that caffeine is degraded to allantoin at low rates in N-deficient plants but not in their N-supplied counterparts. However, this degradation could represent a significant N-source in N-deficient plants. The function of this pathway in *C. arabica* leaves has been previously described [4,7,48].

4. Conclusions

Few studies have been performed using the caffeine from coffee plants, particularly from plants grown in high and low light. In contrast to legumes, little is known regarding the physiological role of ureides in non-nodulated plant species. Thus, we have demonstrated that caffeine is degraded to minimize N-deficiency alterations in the metabolism of coffee plants submitted to N starvation. However, the details of this catabolic pathway are very unclear, and more studies should be developed to elucidate this mechanism. Light may indirectly affect N uptake ($N\text{-}NO_3$ or $N\text{-}NH_4$) from the soil and, for photosynthesis, stimulate glutamine synthetase transcription and activity, resulting in a higher N content in the leaves of plants grown in HL versus to LL environments. The total N content in the leaves is unique for a particular treatment. Thus, when the caffeine content was normalized to these N pools, we observed that the %N allocated to caffeine biosynthesis did not significantly change in the leaves because the N supply was sufficient for the coffee plants (approximately 14%). In contrast, in the LN plants under HL, the value was 9.2%. This value was higher compared to that of the plants grown in shade conditions, which was 7.4%. In particular, under a low N supply, light stimulates caffeine biosynthesis. However, it cannot be assumed that the effect of the environment is independent of the action of genes; our results clearly demonstrated that the N-supply stimulates the biosynthesis of caffeine, e.g., in HL under a low N-supply. Additionally, as nitrogen resources decrease, ALN levels increase, supporting the idea that caffeine may be used as an N-source in N-deficient plants.

Acknowledgements

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE; Grants APQ-0150-2.03/08) for financial support.

Conflict of interest

The authors declare that there are no conflicts of interest related to this study.

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